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ANTIPROLIFERATIVE PROPERTIES OF ETHANOLIC AND AQUEOUS GRAVIOLA LEAF EXTRACTS ON TONGUE SQUAMOUS CELL CARCINOMA CELL LINE-25.

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Abbreviations:

ACGs=acetogenins, AE=Graviola Aqueous Extract, DMSO= Dimethyl Sulfoxide, EE=Graviola Ethanolic Extract, EtOH=Ethanol, GLE= Graviola Leaf Extract, GI_{50}= Growth Inhibition 50%, SCC= Squamous Cell Carcinoma.

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Abstract

Background: *Annona muricata,* commonly known as Graviola, soursop or guanabana, is an evergreen tree native to the tropics with a long history of use in ethnomedicine in indigenous communities in Africa and South America. Its active phytoconstituents have provided medicinal benefits against various ailments and diseases such as arthritis, parasitic infection, hypertension, fever, or diabetes. Studies conducted *in vitro* and *in vivo* have concluded that Graviola phytocomponents have anti-cancer and anti-tumor properties. One of the characteristics of cancer cells is their uncontrolled proliferation rate. In that sense, molecules that inhibit cell proliferation offer potential therapeutical benefits.

Methods: We prepared ethanolic and aqueous extracts from dried Graviola leaves and tested their respective antiproliferative activities on tongue Squamous Cell Carcinoma cell line-25. We treated the cells with increasing concentrations of the extracts for 24 h. The respective doses leading to a 50% inhibition of cells growth (GI_{50}) were determined.

Results: Our results showed that the ethanolic extract was 4 times more active in inhibiting the growth of Squamous Cell Carcinoma cell line-25 than the aqueous extract (respective GI_{50} of 61.7 μg/mL, and 274.6 μg/mL).

Conclusion: We hypothesize that some organic compounds involved in the antiproliferative/cytotoxicity of Graviola leaves were selectively extracted by Ethanol. Future plans include characterizing those bioactive compounds and assessing their activity on Squamous Cell Carcinoma cell line-25 vs. non-cancerous oral cells. Our hope is to discover natural molecules to be used as alternative treatments for oral Squamous Cell Carcinomas.

Keywords: Squamous Cell Carcinoma-Graviola-phytocompounds extractions-antiproliferative molecules- drug discovery.

Materials and Methods:

Preparation of Graviola leaf extracts (GLE) and solubility assays: Leaves from an *Annona muricata* (Graviola) specimen tree in Boquerón, Cabo Rojo, Puerto Rico (coordinates: 18 ° 05'11.9 "N 67 ° 08'44.6" W) were collected. A voucher was deposited at the Mayagüez herbarium of the University of Puerto Rico (Voucher: MAPR, Céline Cassé, 2). The leaves were kept at 4 ° C for preservation. They were first washed with running water and soap, then, with 4% sodium hypochlorite and deionized water, and finally, dried, and cut into small pieces before lyophilization. The samples were "freeze-dried" for three consecutive days (Labconco /Lyph Lock 4.5 lyophilization system, Labconco Corp. ™, Kansas City, MO), milled, and vacuum-stored at -20 ° C.**.**

EE Protocol: EE were prepared by macerating 10 g of powdered Graviola leaves in 100 mL of pure Ethyl Alcohol-200 anhydrous (Sigma Aldrich , St Louis, MO) for 42 hours. The resulting mixture was vacuum-filtered (qualitative filter paper, grade # 415, VWR ™, Radnor, PA). The solid parts were discarded and the filtrate was then passed through a 0.22 μm, 25mm PES filter membrane for sterilization (Membrane Solution ™, LLC, Auburn, WA). GLEs were aliquoted and stored at -20 ° C before use.

AE Protocol: AE were prepared by immersing approximately 10 g of pulverized GL in 75 mL of distilled water (1.3 mg/mL). The leaves were decocted in a 50^{o}C-water bath for 15 minutes. The water was then decanted and preserved for further filtration. This protocol was repeated a total of four times. The decanted GLE was centrifuged, the supernatant collected, and subsequently vacuum-filtered (Qualitative Filter Paper, Grade # 415, VWR ™, Radnor, PA), then frozen and lyophilized. Once lyophilized, the samples were crushed and stored in desiccator chambers.

Growth Inhibition assays: Stocks solutions of EE, EA, and positive control Camptothecin (Sigma Aldrich, St.Louis, MO) were prepared under sterile conditions and diluted in their respective vehicle solvents: 0.1% Dimethyl Sulfoxide (DMSO) for Camptothecin and 0.1% Ethanol for EE. Cells treated with each vehicle alone were used as negative controls. The Squamous Cell Carcinoma cell line-25 (SCC-25) (ATCC CRL-1628, Manassas,VA) was cultured and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5mM L-glutamine, 15mM HEPES, 0.5mM sodium pyruvate and supplemented with 400ng/ml hydrocortisone (Sigma Aldrich, St.Louis, MO), and 10% fetal bovine serum (ATCC, Manassas,VA). SCC-25 cells were maintained at 37^{o}C in a humidified atmosphere of 95% air/ 5% CO_2. Prior to treatments with GLEs, the cells were seeded in 96-well-plates (NUNC, Rochester, NY) at an approximately density of 30,000 cells/well and left to attach for 24 h. They were then exposed to EE or EA at concentrations from 0 to 100 μg/mL (in triplicates) for 24 hours. For cell viability assays, SCC-25 cells were incubated for 30 minutes with 10μL of Prestoblue reagent (Thermo Fisher Waltham, MA). Reduction of the Prestoblue reagent compound generates a fluorescence product only in viable and metabolically active cells. Fluorescence levels were assessed at 544/590nm with the Fluorostar Optima fluorescence reader (BMG lab tech, Cary, NC). Curve fit and GI_{50} dose were obtained by using the fluorometer MARS software data analysis suite (BMG lab tech, Cary, NC). Reported results include only experiments with correlation coefficients $\geq$ 0.95.

Water-solubility protocol: In order to assess the solubility of EE in cell culture media, 10%, 1%, and 0.1% (v:v) dilutions of EE were made in distilled water. The dilutions samples were then centrifuged at 5,000rpm

for 5 minutes. The presence of a pellet corresponding to the insoluble components of the GLE was visually assessed.

Microscopy images: SCC-25 cells were exposed to 100 µg/mL of either Camptothecin, EE, EA. Vehicles solvents alone were used as controls. Plates were seeded at a density of 50,000 cells/mL in 96-well-plates and incubated under the conditions mentioned above. After exposure the cells were observed under a phase-contrast FSX 100 inverted microscope (Olympus Americas, Center Valley PA) at a 10x magnification.

Results:

Water-solubility assays of EE: Preparing botanical extracts involves extracting the plant compounds with an extraction solvent. The most widely used extraction solvents are Acetone, n-Butanol, Chloroform, Dichloromethane, Ethanol (EtOH), Petroleum Ether, Ethyl Acetate, Hexane, and Methanol. In situation where the extraction solvent is poorly miscible in water-based media, a vehicle solvent is used to facilitate the solubilization of the extracted compounds in water.

Even though there is no consensual method for preparing botanical extracts, the solvent used for soaking the plant material is often subsquently eliminated and the extract concentrated to dryness by low-pressure evaporation/rotary evaporation. Such a method offers the advantage of obtaining a weighable solid extract that can be easy stored and preserved (Cunha et al., 2017; Nik Mat Daud et al., 2016; Yang et al., 2015). While experimenting with methanolic and ethanolic extraction process, we dried the GLEs by rotary evaporation. As a result, we obtained a solid paste that was insoluble in organic solvents (data not shown). Instead of concentrating the extract to dryness, we accordingly opted for a simplified protocol in which the compounds are maintained in their original extraction solvent.

Since cells culture media are water-based solutions with pHs close to neutrality, the molecules which activities are to be tested must be water-soluble. In addition, in order to reduce experimental errors due to mortality in controls, the vehicle solvent must be used at a concentration that is non-toxic to the tested cells. Unfortunately, such a rule is not always observed as many solvents used for botanical extractions are cytotoxic at the doses and length of treatment tested: for example, a dilution of DMSO at 0.6 % (v:v) administrated to SCC-25 for 48h has been reported to be associated with a 40% mortality in SCC-25 cells (Nguyen et al., 2016).

EtOH is one of the few organic solvents capable of extracting a good range of polar and non-polar phytocompounds with the advantage of being poorly toxic to cells as a vehicle solvent, even at doses as high as 1% (Nguyen et al. 2015, Liu et al.2008)**.** It has been reported that mixture of [EtOH/water] (7:3)(v:v) are good options for solubilizing botanical molecules (Liu, 2008). Accordingly, we conducted solubility assays of 3 sets of phytocompounds respectively extracted with either **1)** pure EtOH, **2)** 70% EtOH (7:3) (v:v) or **3)** 50% EtOH l (1:1) (v:v). Serial dilutions of the 3 samples were made in water at respective concentrations of 10%, 1%, and 0.1% (v:v). The diluted samples were centrifuged and the formation of a pellet was visually assessed. Our results indicated that a pellet was observed in extractions **1)** and **2)** (data not shown).This indicates that some phytocompounds extrated by mixtures of [EtOH/water] were not water-soluble.

The results of the solubility tests for pure EtOH is presented in **Fig.1**. As shown in **Fig. 1B,** no visible pellet formations were observed after the diluted samples were centrifuged, thus indicating that the extracted

phytocompounds were soluble at all dilutions tested. Due to its solubilization ability and non-toxic properties, pure EtOH was used as the solvent for EE preparation. EE were diluted at a low/non-toxic concentration of 0.1% (v:v) in cells media for growth inhibition assays.

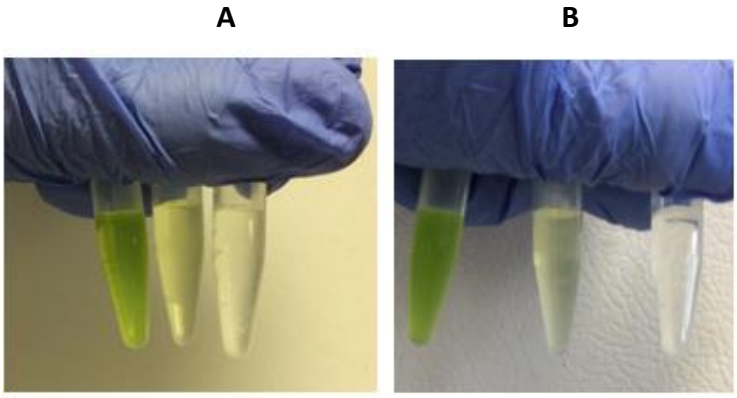

Figure 1. Water- solubility of Graviola leaf compounds extracted with pure Ethanol

Samples were diluted in water at respective concentrations of (from left to right) 10%, 1%, and 0.1% (v:v),**(A)** and the samples were centrifuged at 5,000 rpm for 5 min **(B)**. No pellet formations were observed, thus indicating that EE was water-soluble at all tested dilutions.

Half Growth Inhibition (GI_{50}) assays: The antiproliferative properties of the GLEs were assessed by experimentally determining the dose inducing 50% of cell death (GI_{50}) for EE and EA. Camptothecin, an alkaloid molecule with antiproliferative properties was used as a positive control for bioactivity. The dose-response analysis indicated that the GI_{50} for Camptothecin was 30.0 µg/mL. GI_{50} for EE and EA were respectively of 61.7 µg/mL and 274.6 µg/mL **(Table 1).** Our results indicated that EE was about 4 times more bioactive than EA.

	Camptothecin	EE	EA
GI_{50} (µg/mL)	30.0	61.7	274.6

Table 1. Comparison of bioactivity of Camptothecin, and GLEs on SCC-25 cells.

A dose-response study of the growth inhibition of the SCC-25 cells. Cells were treated with increasing concentrations of either Camptothecin, EE or EA for 24 h and the doses responsible for the death of 50% of cells (GI_{50}) were determined in each case.

We also visually assessed the changes in SCC-25 cells growth after treatment with **(T)** or without **(N)** 100 μg/mL of Camptothecin or GLEs (**Fig.3).** In negative/untreated controls SCC-25 cells have grown to confluence **(Fig.3. N1, N2, N3)**. As expected Camptothecin at 100 μg/mL promoted growth inhibition in SCC-25 **(Fig.3. N1/T1).** Vehicle solvent EtOH alone did not exert any significant antiproliferative effect on SCC-25 **(Fig.3. N2/N3**). There was a weak growth inhibition of SCC-25 cells when treated with EA **(Fig.3. N3/T3**). EE presented a notable antiproliferative activity on SCC-25 cells at 100 μg/mL **(Fig.3. N2 /T2**). Such results were consistent with the respective IG_{50} values obtained for EE and EA.

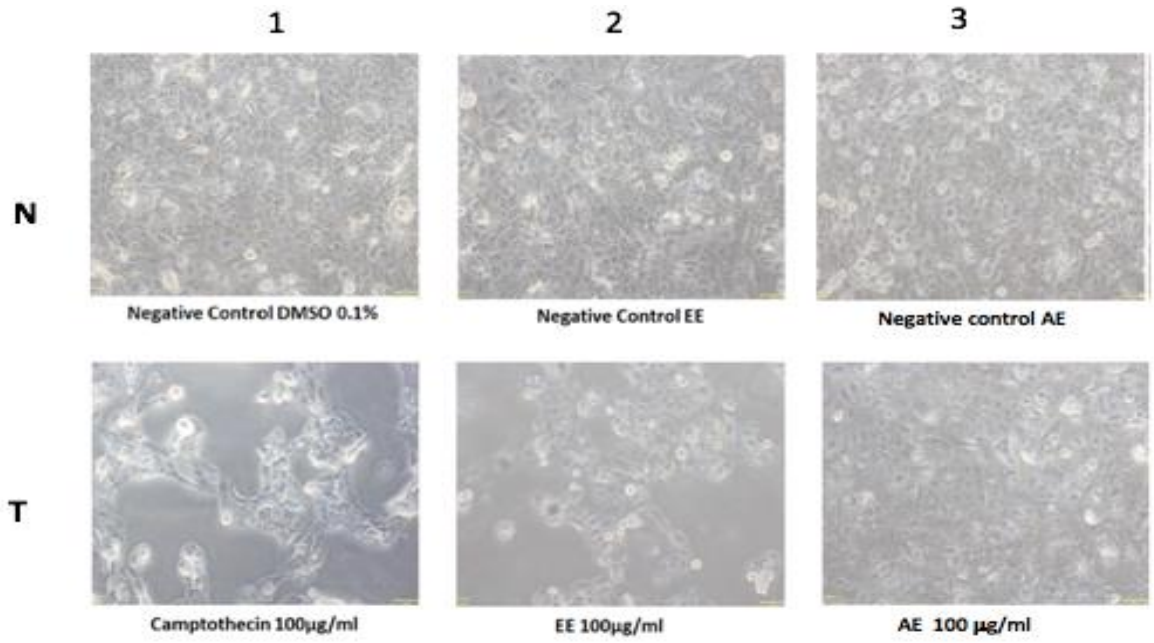

Figure 3. Phase contrast

microscopy images of SCC-25 cells treated or untreated with Camptothecin or GLEs for 24 h. (**N1)-** SCC-25 cells treated with vehicle solvent 0.1% DMSO alone, (**N2)**- SCC-25 cells treated with vehicle solvent 0.1% EtOH alone, (**N3)**- SCC-25 cells in culture media alone. (**T1)-** SCC-25 cells treated with 100 μg/mL Camptothecin. (**T2)-** SCC-25 cells treated with 100 μg/mL of EE in vehicle solvent 0.1% EtOH, (**T3)**- SCC-25 cells treated with AE. Pictures were taken at 10x magnification. Treatment with EE was associated with a significant cells growth inhibition.

Discussion:

Our results indicate that AE have a limited bioactivity (IG_{50}> 200 μg/mL). This result contradicts a former study by Magadi in which a much stronger bioactivity for AE (12 μg/mL) was reported after a 24h treatment (Magadi et al., 2015). Such a discrepancy could be attributed to: 1) some variations in the phytocomposition of the specimens leaves due to pedological and geographical differences, 2) the differences in the extraction protocols between the two studies (decoction at 50 ^{0}C in this study vs. Soxlet/reflux technique for Magadi et al.).

Comparision between bioactivity of EE and AE shows that that EE is 4 times more active than EA. To explain such a result, we hypothesize that EtOH solubilizes a broader variety of phytocompounds than water, some of which, such as ACGs have antiproliferative properties (Cassé, 2018) . The bioactivity of a botanical extract

depends not only on the individual properties of its individual family of phytocompounds but could result from them working synergistically. A study conducted on an *in vivo* model for prostate cancer shows that while the ACGs-enriched extract appear to be the most efficient to induce tumor-suppression, the presence of the flavonoids in the whole leaf extract modulates the cytotoxicity of ACGs and confers possible maximum therapeutic benefits (Yang et al., 2015).

Future plans include pharmacokinetics studies of EE stability, characterizing the molecules responsible for EE antiproliferative properties and assessing the molecular mechanisms leading to SCC-25 cells death. By doing so, we hope to better understand the role of individual compounds and the way those phytocompounds interact to promote cell growth inhibition in oral Squamous Cell Carcinoma.

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